APMIS *Suppl.* 27, vol. 100: 71-83, 1992

EXHIBIT "J"

Microbial interaction with animal cell surface carbohydrates

KARL-ANDERS KARLSSON, JONAS ÅNGSTRÖM, JÖRGEN BERGSTRÖM and BOEL LANNE

Membrane Glycobiology Group, Department of Medical Biochemistry, University of Gothenburg, Sweden

Karlsson, K.-A., Ångström, J., Bergström, J. & Lanne, B. Microbial interaction with animal cell surface carbohydrates. APMIS *Suppl.* 27, vol. 100: 71-83, 1992.

Microbes have selected primarily carbohydrates for attachment to host animal cells. Recent studies have revealed essential characteristics in the recognition of receptor carbohydrates. Of importance is the property of recognizing also sequences placed inside an oligosaccharide chain, which differs from most animal antibodies. This is the basis for series of isoreceptors with the minimum receptor sequence in common but with separate neighbouring groups. There are families of microbial ligands that show different preferences for members within one series of isoreceptors, indicating only slight differences in the complementary binding sites of the proteins. Such differences may explain shifts in the selectivity of separate host tissues for infection. A second characteristic is the low affinity interaction often found where simple receptor-containing saccharides are unable to inhibit attachment. Technical possibilities are rapidly developing for the design of synthetic receptor analogues to be used in the therapy of clinical infections. This is urgently needed in cases where no rational therapy exists today.

Key words: Microbe; bacteria; virus; toxin; carbohydrate; glycosphingolipid; receptor; adhesion; binding epitope; epitope dissection; adhesin; therapy.

Professor K.-A. Karlsson, Membrane Glycobiology Group, Department of Medical Biochemistry, P.O. Box 33031, S-400 33 Gothenburg, Sweden.

The abundance of carbohydrates in various forms at the animal cell surface is one reason why microbes to a large extent have selected carbohydrate receptors for colonization and infection. Glycolipids play a special role since they are usually strictly membrane-bound and do not appear in secretions as potential inhibitors of membrane attachment, as do glycoproteins. They are also easier to handle technically than protein-bound oligosaccharides as there is only one oligosaccharide for each molecule. This allows a rational binding assay based on overlay of a virus (8) or bacterial (9) suspension on thin-layer chromatograms with separated molecular species of glycolipids. Many microbes have, in fact, selected relatively glycolipid-specific receptors (lactose, and glycolipid members of the ganglio and globo series). The development during the last few years of assay, separation and structural techniques for glycoconjugates, in combination with knowledge from microbial molecular biology, is at present contributing information on both prokaryotic and eukaryotic protein-carbohydrate interactions that hold promise also for drug design (14).

The purpose of the present communication is to summarize and discuss important characteristics of glycolipid receptors for microbes with emphasis on recent results from our own laboratory and extrapolation to biomedical applications. The recognition by microbes of internal sequences of the saccharide chain and the potential membrane proximity of the binding are two properties of more conceptual interest.

THE GENERAL PROPERTY OF MICROBES TO RECOGNIZE NON-TERMINAL RECEPTOR SEQUENCES IS THE BASIS FOR FAMILIES OF RECEPTOR-BINDING VARIANTS

In contrast to animal antibodies that recognize carbohydrates, almost all of which bind to terminal sequences (31), microbial proteins have been convincingly shown to bind also to internal epitopes of receptor saccharides (13, 18). This is true for viruses, bacteria and bacterial toxins and this property will appear as one characteristic in the following presentation.

When using the rational overlay techniques (8, 9, 15) to detect glycolipid-based receptors, a particular ligand may bind to a large number of receptor-active bands on the thin-layer plate (3). Of these, only a few may have the active sequence in the terminal position (3). Also, some glycolipids which carry the active sequence in internal position may be receptor-inactive, based on non-accessibility of the binding epitope due to steric hindrances from neighbouring groups or for other reasons. We have defined the name *isoreceptors* for oligosaccharide sequences, regardless of whether or not they are active for a particular ligand, which have the minimum receptor sequence in common but placed in different environments (flanked by separate neighbouring groups). A large number of bacteria, bacterial toxins and viruses have already been analysed using this overlay technique. An interesting grouping of receptor specificities into families has been revealed (13, 18), where each family consists of ligands that require the same minimum receptor sequence but where individual members may show different preferences for individual isoreceptors. Examples of such families are given in Table 1.

Within each family a minimum sequence is required but there is separate dependence on flanking sequences or ceramide structure. The conclusion has been that the binding epitope on the isoreceptors varies slightly between variant binders and that the complementary binding sites of the receptor-binding proteins are structurally and evolutionary related.

The biological meaning of the internal binding and these variants has been briefly discussed (13), and there is increasing experimental evidence to support the given proposal. The mutational effort to shift specificity between two isoreceptors was considered limited (a few amino acids in the binding site) compared to a shift between two families of isoreceptors. The internal binding is essential as the basis for isoreceptors

TABLE 1. *Families of receptor-binding variants of microbial ligands. Within one family, variants show different binding preferences to isoreceptors, which have a minimum receptor sequence in common. The information has been taken mainly from Refs. 2, 13, 18, 19, 26, 27, 28, 29.*

Lactosylceramide binders R1-*Galβ4Glc*-R2	A large number of bacteria have been shown to bind to separate patterns of isoreceptors with lactose as minimum requirement. The bacteria are both normal flora, mainly large intestine, and important pathogens such as *Bordetella pertussis, Vibrio cholerae, Shigella dysenteriae, Neisseria gonorrhoeae.* Among the isoreceptors are lactosylceramide species with separate ceramide structures and these are illustrated in the present paper (Table 3).
Galabiose binders R1-*Galα4Gal*-R2	Several variant adhesins of different *Escherichia coli* strains causing urinary tract infection in human and dog. Shiga-like toxins.
Sialic acid binders R1-*NeuAc*-R2	Although not yet established, this is probably a very large family, including several viruses, bacteria and bacterial toxins. The illustration in the present paper shows cholera and tetanus toxins, and their different but related binding epitopes on isoreceptors (Fig. 8).
Monoglycosylceramide binders R1-*HexβCer*	A group of viruses have been shown to bind with this specificity. These belong to *Adenoviridae, Herepetoviridae, Orthomyxoviridae, Paramyxoviridae, Myxoviridae, Rhabdoviridae, Reoviridae* and *Retroviridae*, including for example influenza and AIDS virus. The two binding variants Sendai virus and HIV-1 will be illustrated in the present paper (Table 6).

TABLE 2. *Relation between different binding to isoreceptors on cell membranes and tissue tropism in urinary tract infections in human and dog. Data are taken from Refs. 3, 18, 20, 21 and 29. For isoreceptor sequences, see Figs. 1 and 2.*

	Globoside	Forssman	Globo A
Binding by			
Class II adhesin	+	–	–
Class III adhesin	–	+	+
Isoreceptor presence in urinary tract of			
Dog	–	+	?
Blood group A-positive humans	+	–	+
Blood group A-negative humans	+	–	–
Binding to epithelial cells of human urinary tract when *only* Class III adhesin is expressed by *E. coli* isolate			
Blood group A1 secretors	–	–	+
Blood group A1 non-secretors, blood group A2 or blood group A-negative	–	–	–

	Class II Adhesin	Class III Adhesin
Frequency of expression of adhesin in *E. coli* isolates of		
Human	+++	+
Dog	+	+++

with only subtle differences at the binding epitope; a terminal binding, with a neighbour only on one side, would most probably require a greater effort for a shift to occur. Also, the main difference between isoreceptors lies within the neighbour most distal from ceramide. To have biological significance, it must be possible to correlate this main characteristic of microbes with virulence properties. In the following, two examples will be shown where a shift in tissue tropism may be explained on the basis of variant binders. The first is *E. coli* and urinary tract infection and a shift between human and dog, and the second is lactosylceramide binders and epithelial and non-epithelial localization. The first will now be discussed, and the second later in this presentation.

Urinary tract infection and shift in tropism between human and dog

As summarized in Table 2, there is an interesting finding where a change in colonization between human and dog may be explained by a receptor shift beween two isoreceptors within the galabiose family (Table 1). In the first detailed characterization of a carbohydrate-binding specificity for a bacterium published in 1985 (3), a clinical isolate of *E. coli* from human urinary tract infection was shown to recognize on thin-layer plates all known isoreceptors within the galabiose glycolipid family (globo series of glycolipids). It has later been found through extensive work on cloning variant adhesins and testing these for receptor specificity combined with frequency of expression in urinary tract infection (29), that a shift in binding between globoside (Class II adhesin) and Forssman glycolipid (Class III adhesin) may explain colonization in the urinary tract of human and dog, respectively. Furthermore, it has been shown that human isolates of *E. coli* that express only Class III adhesin appear in individuals of blood group A only (20). This may be explained (18, 29) by a dominance of globoside (binding by Class II adhesin) in the human and a dominance of Forssman glycolipid (binding by Class III adhesin) in the dog. The difference in binding epitope between Class II and Class III adhesins is probably that Class II requires Galβ in GalNAcβ3Galα4Galβ, while Class III has an epitope shifted to the left and not requiring Galβ. This is illustrated in Fig. 1, where calculated conformations of the molecules as they probably appear in the surface membrane are projected for globoside and Forssman glycolipid (left and right, respectively) with indicated arbitrary binding epitopes. The reason why Class II does not bind to Forssman glycolipid is that the Galβ

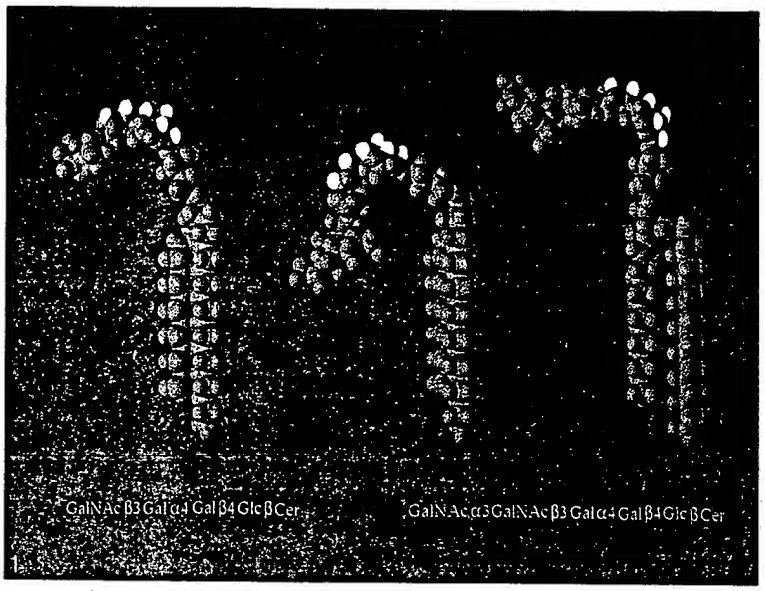

Fig. 1. Molecular models of globoside (left), and Forssman glycolipid (in two separate conformations). The non-polar ring hydrogens of Galα4Gal are shown in white and the rest of the molecules in grey. The purpose is to illustrate the probable basis for the tropism of urinary tract infection in human and dog, respectively (compare with binding data of Table 2). The Class II adhesin of *E. coli*, which is most frequent in human infections, binds selectively to globoside on cell membranes. The Class III adhesin, however, which is most frequent in dog infections, binds selectively to Forssman glycolipid. Globoside is typical of human cells and Forssman glycolipid of dog cells. Interestingly, this discrimination is not present when the two glycolipids are exposed on assay surfaces like thin-layer plates or microtitre wells. The explanation is that the slightly different binding epitopes for the two adhesins are present on both glycolipids but selectively hidden when placed on the membrane, though not on the assay surface. The models show that the Galα4Gal, which is required for Class II adhesin, is easily accessible in globoside (left) but not Forssman glycolipid (far right), when they are placed in the membrane. However, on assay surfaces, without restrictions from placement in the membrane, the conformation in the middle (one of several) is also possible, where the disaccharide is better presented. This demonstrates that membrane proximity of the binding epitope may influence the specificity. For modelling, see data in Table 4.

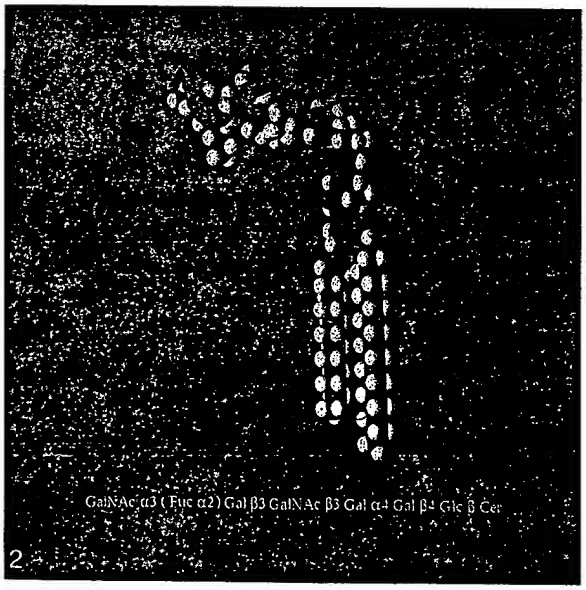

Fig. 2. Molecular model of blood group A globoside which is selectively recognized on cell membranes by Class III adhesin of *E. coli* in urinary tract infection (compare Table 2). It may be compared with the far right conformation of the Forssman glycolipid of Fig. 1. Although the binding epitope for Class II adhesin exists in this glycolipid, it is selectively shielded by the bending of the saccharide in the membrane location, compared with the non-membrane location (compare middle conformation in Fig. 1).

residue is forced down at the side in the Forssman glycolipid (and in the globo A glycolipid, which is visualized in Fig. 2) and is thus not accessible for binding. Class III, on the other hand, not requiring this Galβ (and not able to bind to sequences with terminal Galα4Gal, in contrast to Class II, see References 18 and 29) binds perfectly to this conformation. Why Class III practically does not bind to globoside in the membrane (but binds well on technical assay surfaces, see below) may be that the Class III epitope (as it exists on Forssman or globo A) is not properly presented.

The conclusion from this discussion is that the tropism of infection may be shifted by a minimum change in the binding site of the adhesin (not yet localized in the cloned sequences), and that this determines a change in selectivity for isoreceptors on the target cell membrane. A different appearance of isoreceptor patterns between cells, which is often found, is the basis of the targeting to specific cells.

THE DEPENDENCE OF SPECIFICITY ON CERAMIDE STRUCTURE: MEMBRANE PROXIMITY AND CONFORMATION

Galabiose series of receptors

The proximity of a carbohydrate binding epitope to ceramide may have effects on the selectivity of microbial binding to membrane-located glycolipids due to the constraints produced by the bilayer surface. These constraints are not present when the glycolipid appears in a non-membrane environment, as on technical assay surfaces. A convincing example of this exists within the galabiose family of binders (see the discussion above). Class II adhesin binds about equally well to globoside and to Forssman glycolipid or globo A glycolipid when they are presented on thin-layer chromatograms or in microtitre wells. However, on intact cells only globoside is recognized (29). A reason for this is illustrated in Fig. 1, where the Forssman glycolipid is presented in two low-energy conformers, one of which may exist on non-membrane surfaces but is unlikely to exist in membranes (middle), and one of which may exist in the membrane (right). If Class II adhesin is dependent on accessibility of Galβ in galabiose for binding, this part will then be exposed in the centre conformer but not in the right one due to the bending of the saccharide forced by the membrane location. In the right conformer this sequence is usually presented to the side in the membrane. Thus the membrane location produces a higher selectivity in binding to glycolipids than when these appear on technical surfaces. Logically, the presentation that the bacteria select for an optimal colonization should be the membrane-dependent presentation on target cells. The membrane proximity may also be important for penetration (see discussion below on second-step receptors).

Lactosylceramides

An example of dependence of epitope conformation on ceramide structure regardless of membrane location, is the family of lactose binders (Table 1). As shown in Table 3, there are two groups of members depending on which type of lactosylceramide they recognize. Interestingly, this may be related to

TABLE 3. *Members of the lactosylceramide-binding family (Table 1) that select lactosylceramides, Galβ4GlcβCer, based on differences in ceramide structure. Data taken from Refs. 11, 13, 18, 27 and 28.*

	LacCer with sphingosine and non-hydroxy acid	LacCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid
Propionibacterium freudenreichii and several yeasts and fungi	+ +	–
Normal flora of large intestine and several pathogens, see Table 1	–	+ +

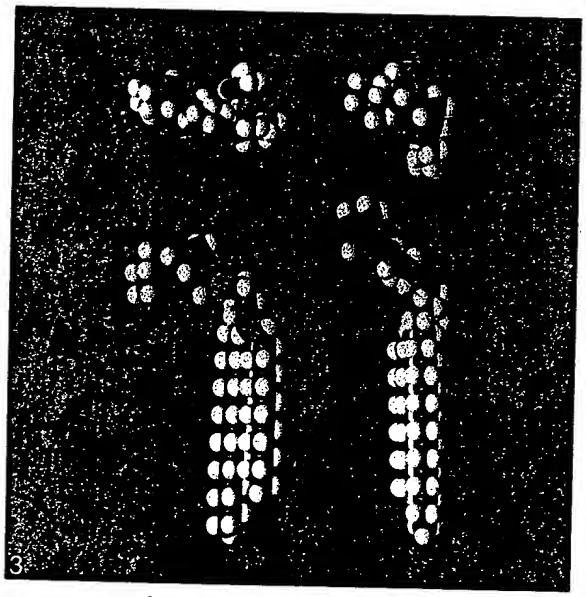

Fig. 3. Molecular models of low-energy conformers of lactosylceramide having separate ceramide structures and being selectively recognized by microbes (compare with binding data of Table 3). The left species is LacCer with C16 sphingosine and 2-D-hydroxy 18:0 fatty acid, and the right species LacCer with C16 sphingosine and non-hydroxy 18:0 fatty acid. O-6 of Gal and Glc have been labelled with a purple colour, and as can be seen O-6 of Gal is not accessible for binding in the left conformer in contrast to the right conformer. (This is best seen in the two top projections which show the surfaces of a membrane location which are exposed to the outside). The polar side of Gal is exposed in the left conformer and the non-polar side in the right conformer. It is likely that binding to these two sides explains the selectivity with no cross-binding by the two groups of microbes (Table 3). For modelling conditions, see Table 4.

TABLE 4. *Minimum energy conformers of lactosylceramide and sulfated galactosylceramide with 2-D-hydroxy fatty acid or non-hydroxy fatty acid*[a]

Conformer	Relative energy (kcal/mol)	HexβCer dihedral angles[b] Φ	Ψ	θ	HexβCer hydrogen bonds[c]
LacCer with 2-D-OH acid					
1*	0	13	-90	-59	O-2 ··· HN, OH-2 ··· O'-2
2	5.0	51	-179	67	OH-2 ··· O-2, O-1 ··· HO-2
3[d]	5.1	48	-138	63	OH-2 ··· O-2, O-1 ··· HO-2
4	7.7	47	-74	-172	OH-2 ··· O-1
5[e]	9.0	41	-93	165	OH-2 ··· O-1
6	10.4	51	180	177	–
LacCer with non-OH acid					
1	0	47	177	66	OH-2 ··· O-2, O-1 ··· HO-2
2	1.8	15	-90	-61	O-2 ··· HN
3*	2.2	50	-74	-176	OH-2 ··· O-1
4[f]	2.7	47	-122	65	OH-2 ··· O-2, O-1 ··· HO-2
5	3.7	40	-97	167	OH-2 ··· O-1
6	4.6	48	177	175	–
SO_3-3GalCer with 2-D-OH acid					
1*	0	12	-90	-59	O-2 ··· HN, OH-2 ··· O'-2
2	2.3	13	-91	-59	O-2 ··· HN, OH-2 ··· OS
3	2.7	56	-178	68	O-1 ··· HO-2, OH-2 ··· OS
4	4.0	39	-158	67	OH-2 ··· O-2, O-1 ··· HO-2
5	5.5	53	176	180	OH-2 ··· OS
6	5.7	54	-177	-63	OH-2 ··· OS
SO_3-3GalCer with non-OH acid					
1	0	14	-91	-60	O-2 ··· HN, OH-2 ··· OS
2	0.7	56	179	69	O-1 ··· HO-2, OH-2 ··· OS
3	2.8	50	173	-55	OH-2 ··· OS
4	3.1	43	-156	-66	OH-2 ··· O-2, O-1 ··· HO-2
5	4.3	52	179	178	OH-2 ··· OS
6*	4.7	46	-75	-169	OH-2 ··· O'-1

[a] The relative energy of the minimum energy conformers of GalβCer is the same as listed above for LacCer. Minimum energy conformers of the various molecules shown in Figs. 1-5 and 8 were calculated using the DREIDING force field (23) within the Biograf molecular modelling software package from Molecular Simulations Inc. placed on a Silicon Graphics 4D/25TG workstation.

[b] The hexose-ceramide dihedral angles are defined as follows: Φ = H-1 - C-1 - O-1 - C-1, Ψ = C-1 - O-1 - C-1 - C-2, and Θ = O-1 - C-1 - C-2 - C-3.

[c] Hydrogen bonds are listed with the hexose donor (acceptor) first. In several cases, however, the OH-2 of Gal preferably forms a hydrogen bond with the sulfate group in the sulfatide, which for the sake of completeness is also given. Hydrogen bond energies are in the order of -5 to -8 kcal/mol. Substituents on the fatty acid have been primed.

[d] Strained conformer of no. 2.

[e] Strained conformer of no. 4.

[f] Strained conformer of no. 1.

* Denotes conformers shown in Figs. 3-5.

the tropism of infection, since lactosylceramide with less hydroxylated ceramide is present in non-epithelial cells, while that with the higher level of hydroxylation is present in epithelial cells lining the mucous surfaces. The level of hydroxylation has been proposed to affect membrane stability (12). There is no evidence that the ceramide is part of the binding epitopes. It is more likely that the lactose moiety shows different sides for binding, as depicted by molecular modelling in Fig. 3 (see also Table 4 for energy levels). Consider for example OH-6 of Gal, which is available for binding in the low-energy conformer of the less hydroxylated species (right), whereas it is pointing downwards in relation to the membrane surface in the conformer of the other species (left). Therefore, when the two conformers are compared, the disaccharide is presented differently to a potential binder, with the non-polar side of Gal available in the right conformer but the polar side of Gal in the left one. It is hence likely that the two groups of ligands recognize separate epitopes on lactose with no cross-binding. Furthermore, the members which selectively bind the hydroxylated species can also bind to several extended sequences (e.g. Galα3, GalNAcβ4, Galβ3GalNAcβ4), which members binding the non-hydroxylated forms are unable to do (13, 27). Within the former group there are variants which show different preferences in binding to extended sequences of lactosylceramide (13, 18).

Hexosylceramides

Using the thin-layer chromatographic overlay method (8) we have demonstrated that several non-related viruses carry a common receptor specificity based on glycolipids (16, 18). Recently, a related specificity was also found for the AIDS virus, HIV-1 (2, 10). The actual viruses are gathered in Table 5.

As shown in more detail for Sendai virus (16, 18), HexβCer may be extended by up to four sugars with retained binding. However, only some extensions are positive, probably due to steric hindrance or other causes of non-accessibility of the binding epitope. Thus, also viruses carry the property of binding internally in a saccharide chain. Except for HIV-1, both GlcβCer and GalβCer are receptor-active. However, there is a dependency on ceramide structure analogous to the case for lactosylceramide binders (see discussion above with respect to Tables 3 and 4, and Fig. 3). Except for HIV-1, the viruses of Table 5 bind exclusively to HexβCer species with sphingosine combined with hydroxy fatty acid or phytosphingosine combined with hydroxy fatty acid. HIV-1, on the other hand, differs by also binding to species with non-hydroxy fatty acid, but only to GalβCer and not to GlcβCer. HIV-1, in addition, binds to sulfatide, which Sendai virus does not. These data are summarized in Table 6. (One reservation should be expressed

TABLE 5. *Viruses that have been shown to carry a common glycolipid-binding specificity with a minimum sequence of one sugar. Data were taken from Refs. 2, 10, 16 and 18.*

Member	Classical receptor	Additional specificity
Adenovirus 2, 7	Peptide	HexβCer
B95-8 EB virus	B-cell peptide	HexβCer
Influenza virus	Sialic acid	HexβCer
Mumps virus	Sialic acid	HexβCer
Sendai virus, two variants	Sialic acid	HexβCer
Rabies virus ERA	?	HexβCer
Rotavirus K8	Carbohydrate	HexβCer
Reovirus 1, 2, 3	Carbohydrate?	HexβCer
HIV-1	CD4-peptide	HexβCer

TABLE 6. *Comparison of Sendai virus (16, 18) and HIV-1 (2, 10) with respect to binding to one-sugar glycolipids using the thin-layer chromatogram overlay method*

	Sendai Virus	HIV-1
GlcβCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid (Left conformer of Fig. 5)	+	–
GalβCer with sphingosine and non-hydroxy acid (Right conformer in Fig. 4)	–	+
GalβCer with sphingosine and hydroxy acid or phytosphingosine and hydroxy acid (Left conformer in Fig. 4)	+	?
Sulfatide (SO_3-3GalβCer, centre and right conformers in Fig. 5)	–	+
Extensions of GlcβCer, eg. Galβ4GlcβCer, Galβ3(Fucα4)-GlcNAcβ3Galβ4GlcβCer	+	–

as to the details respecting these differences: HIV-1 on the one hand, and the other viruses on the other, have been characterized by separate laboratories. Until they have been compared under exactly the same conditions, no definite conclusions can be drawn.)

In the following we will present a preliminary interpretation of probable differences in binding epitopes between Sendai virus and HIV-1, based on calculated low-energy conformers of various isoreceptors (Figs. 4 and 5). The two models, hydroxylated and non-hydroxylated, of GalβCer of Fig. 4 are analogous to those of lactosylceramide in Fig. 3. One conclusion was that part of the ceramide is included in the recognized epitope of the hydroxylated form and that the non-polar side of Hex is involved in the binding of viruses which are not HIV-1 (16, 18). This is visualized in the left conformer in Fig. 4, where both part of the ceramide and the non-polar ring hydrogens of Gal are accessible on the top projection. A reason why also GlcβCer is active may be seen from this conformer, where a change in configuration of O-4 (yellow) to Glc

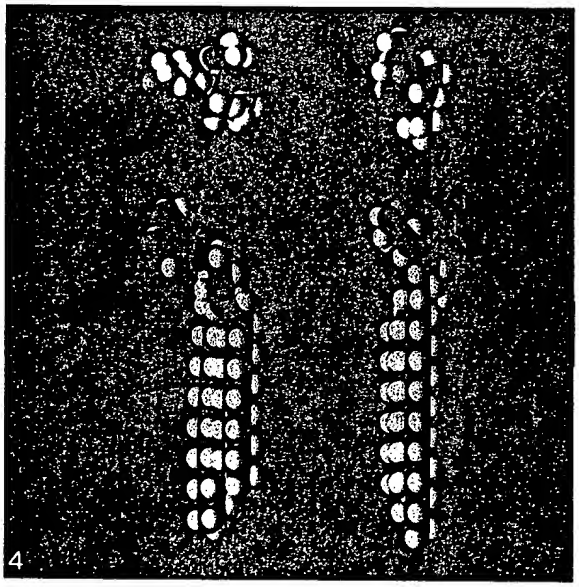

Fig. 4. Molecular models of low-energy conformers of GalβCer having separate ceramide structures, the left being recognized by several viruses which are not HIV-1, and the right being recognized by HIV-1 (compare binding data of Table 6). The two species correspond to the ceramide structures of LacCers of Fig. 3. Viruses that bind the left species also recognize GlcβCer, which is not the case for HIV-1. The O-4, which differs in configuration between Gal and Glc, is indicated in a separate yellow colour, as is O-3 (purple), which is the position for a sulfate group (compare full-colour models of GlcβCer and sulfatides of Fig. 5). One may envisage that O-4 has a more critical exposition in the right conformer and that change to Glc-O-4 may disturb a directed hydrogen bonding. Also, a sulfate group at O-3 is tolerated by HIV-1, which recognizes a polar side of Gal. In contrast, the other viruses probably interact mainly with a non-polar side of Gal or Glc (best seen in the top projection to the left) and do not tolerate a sulfate group in position 3. On the other hand, the configuration of O-4 is critical for HIV-1 but not for the other viruses. Therefore, the two groups of viruses are variants of HexβCer binders. For modelling conditions, see Table 4.

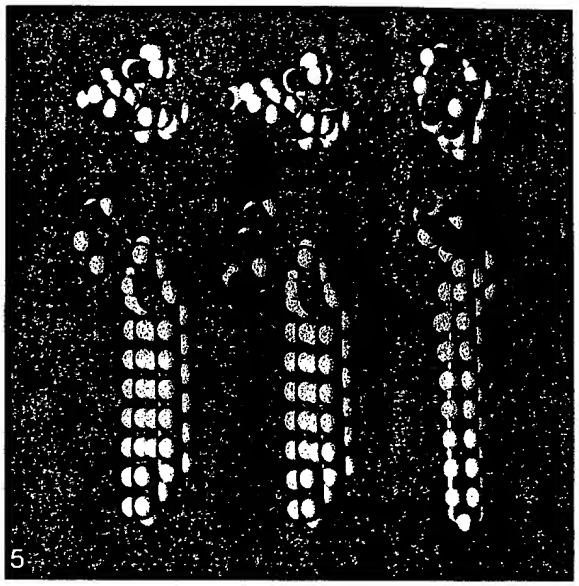

Fig. 5. Low-energy conformers of GlcβCer (same ceramide as GalβCer of Fig. 4, left), and two species of sulfatide, SO_3-3GalβCer, corresponding to the two conformers of GalβCer of Fig. 4. Sulfatide is recognized by HIV-1 but not by the other viruses of Table 5. On the other hand, GlcβCer is not recognized by HIV-1, but by the other viruses. One may envisage that the sulfate group interferes with a mainly non-polar interaction for non-HIV-1 viruses (left conformer of Fig. 4), while it is part of the more polar side being recognized by HIV-1 (compare right conformer of Fig. 4). O-4 on GlcβCer (left) does not interfere with binding by non-HIV-1 viruses and may be extended with further sugars without interfering with binding. In contrast, HIV-1, which may have a directed hydrogen bonding with OH-4 of Gal, does not accept an inverse configuration of this group. Compare legend of Fig. 4. For modelling conditions, see Table 4.

may not be critical for accessibility of the binding side (see also Fig 5, left). On the other hand, in the binding by HIV-1 to the right conformer in Fig. 4, the configuration of OH-4 may be critical, if it, as indicated, is exposed on the binding side and may be involved in hydrogen bonding. A less polar interaction in the case of Sendai virus may explain why sulfatide is inactive (position 3 for the sulfate group is indicated in purple in Fig. 4 and corresponding sulfatide models are shown in Fig. 5). In contrast, HIV-1 may carry a positively charged complementary site that tolerates the sulfate group in a neighbouring position to the postulated hydrogen bonding at OH-4. Thus, Sendai virus and HIV-1 are variant binders to isoreceptors of HexβCer. The biological meaning of this binding will be discussed below in relation to second-step receptors.

SECOND-STEP RECEPTORS BASED ON GLYCOLIPID SPECIFICITY AND MEMBRANE PROXIMITY: A HYPOTHESIS

Lactosylceramide-binding bacteria or HexβCer-binding viruses that colonize or infect separate cells or tissues are so diverse that they certainly cannot use a common binding specificity to effect these various tropisms. A characteristic feature seems to be the presence of at least two binding specificities for each particular microbe (see Table 5). A working model (13, 16) predicts that the infectious process involves a two-step mechanism (or in some bacterial cases a multistep mechanism). The first-step receptor mediates the targeting and tropism of the infection. The second-step receptor establishes a true cell-membrane attachment (lactose binders) or mediates the penetration into cells (some lactose-binding bacteria and HexβCer-binding viruses). For this to work, the second-step receptors used in common cannot be directly accessible from the outside of the cell, since this would destroy the selectivity. Therefore, the accessibility has to be induced after binding to the first-step receptor. Firstly, it has been established that one- and two-sugar glycolipids are not accessible for galactose oxidase labelling on normal cells (5, and discussion in 13). Secondly, a kinetic cell adhesion model (1) includes repulsion forces from an approaching particle (less likely for a molecule) that may induce a lateral diffusion and reorganization of surface components for a final lowest-energy binding. These data therefore support our model.

In the case of lactosylceramide-binding bacteria, it is of interest that lactose in bound form is known only in glycolipids, which are specifically membrane-associated and do not appear in secretions as do glycoproteins, which are potential inhibitors of membrane attachment. Bacteria may therefore secure a true cell adhesion by a second-step lactosylceramide receptor. Furthermore, several of these bacteria are invasive (13) and lactose binding next to ceramide may mediate an essential proximity to the membrane bilayer.

Viruses have to penetrate into the host cell to reproduce. Therefore the second-step binding to isoreceptors of HexβCer may give the proximity between virus and host cell membranes of less than 10 Å to induce membrane fusion, either at the plasma cell membrane or in the endosome. Although the biological relevance of the second-step mechanism has not yet been proven, the recent data from HIV-1 and blocking of both binding and infectivity, and localization of binding activity to gp120, are positive arguments (2, 10).

POTENTIAL APPLICATIONS

It is an old concept that in order to infect microbes have to specifically adhere to the target cell to avoid being eluted. In the case of viruses there is also a need to get into the cell. Applications where inhibition of attachment or adhesion can prevent or cure an infection in cases where no alternative is working in practice seem attractive. In the following some data relevant for anti-adhesion applications will be discussed.

The affinity of interactions

When trying to inhibit attachment there are two levels of inhibition, one where free oligosaccharides are able to inhibit and one where they are not able to inhibit . As an illustration, uropathogenic *E. coli* and Shiga toxin both recognize isoreceptors based on Galα4Gal (3, 19). How-

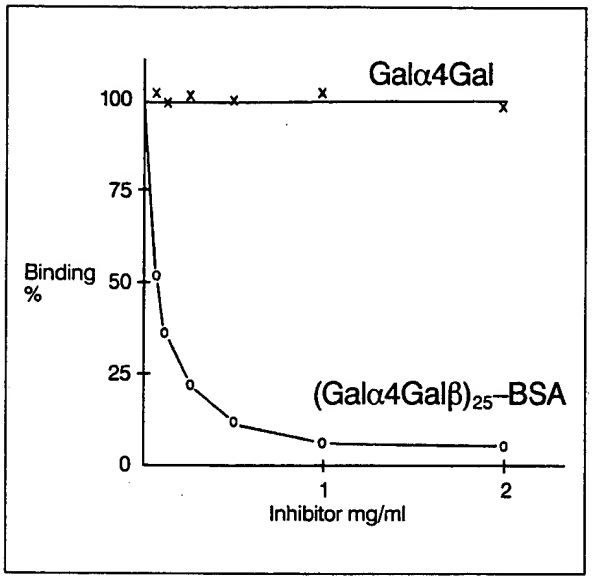

Fig. 6 Binding curves for inhibition of binding of Shiga toxin to Vero cells. Univalent Galα4Gal is unable to inhibit up to 2 mg/ml, while the same disaccharide linked multivalently to BSA (optimal with 25 residues per BSA molecule) is very efficient. Data taken from Ref. 19.

ever, while it is possible to inhibit *E. coli* attachment to target cells by univalent free oligosaccharides, this is not the case with Shiga toxin. As shown in Fig. 6, inhibition of binding of Shiga toxin (six or seven binding subunits) to Vero cells is not possible with relatively high concentrations of oligosaccharide. (At this level *E. coli* is inhibited *in vitro* in its adhesion to uroepithelial cells, although not completely.) However, when multivalently presented in conjugation with BSA, there is efficient inhibition. This is receptor-specific, since lactose linked in the same way is completely inactive. Binding to glycolipid receptors on thin-layer plates or in microtitre wells works well for both ligands due to a multivalent presentation. One conclusion from this is that the traditional inhibition assay or criterion of receptor specificity based on inhibition with free oligosaccharides is not applicable in some microbial protein-carbohydrate interactions. In fact, our impression at the present stage of knowledge is that a majority of systems have this low-affinity type of binding. Simple binding curves from binding of the ligand in microtitre wells coated with receptor-active glycolipid may define whether the ligand is of this type or not (15, 19, 28). The classical NeuAc receptor for influenza virus is of this type. Interestingly, several synthetic chemistry papers have appeared during the last year showing that NeuAc linked multivalently to different carriers are efficient inhibitors of virus binding (6, 7 , 22, 25).

Generally, the interaction between a virus, bacterium or parasite and the animal host cell is multivalent. For an inhibitor to be efficient in competing with this binding, there must be multivalency or, if univalent, the affinity of individual sites must probably be much higher than the natural affinity. For influenza virus, it is possible to inhibit with univalent receptor analogues where NeuAc is linked with a spacer to various aromatic structures (32). The best inhibitor was 64 times better than the methyl ketoside. Similarly, a conceptually important result is the thousand-fold improvement in efficiency of both inhibition of yeast cell agglutination and binding to intestinal epithelial cells of type 1 *E. coli* using aromatic residues linked to Manα (4). As a control, the corresponding Glcα derivative was completely inactive, showing that it is possible to improve an analogue this much with retained binding specificity. In this case it was even possible to elute already adhered bacteria.

In principle, a low-affinity interaction should mean an inefficient fit between protein and carbohydrate. Theoretically, it is possible to design an analogue with perfect fit to improve the interaction (Fig. 7). In cases with extremely high

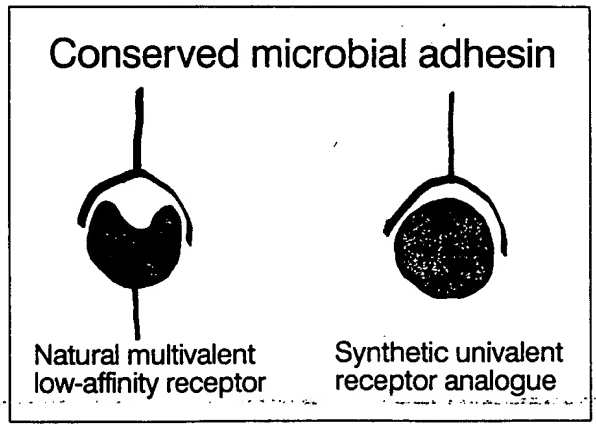

Fig. 7. Figure to visualize the assumed non-perfect fit of carbohydrate receptor and protein site (left) in case of low-affinity interactions (of the type shown in Fig. 6). On the right, an optimized synthetic analogue is visualized to have a better fit and thus be a much better binder than the natural receptor structure.

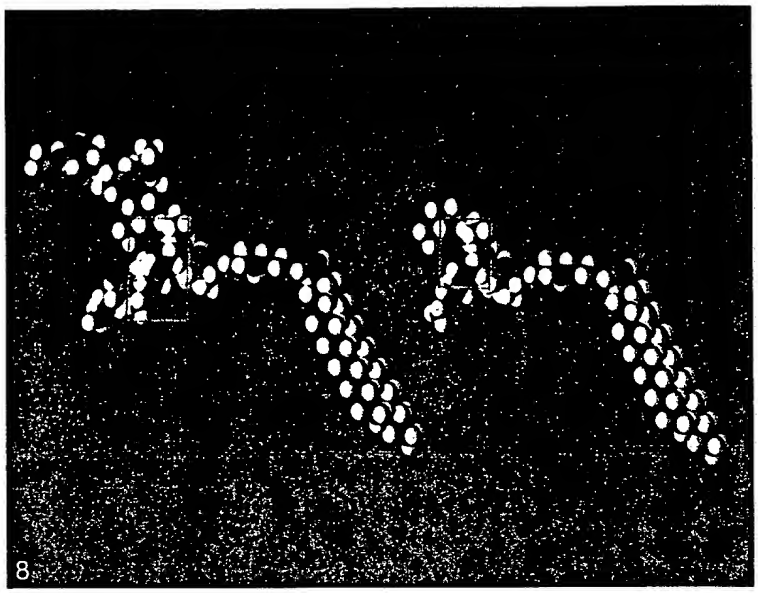

Fig. 8. Molecular models of the gangliosides GM1 (right) and GQ1b, which are optimal receptors for cholera toxin and tetanus toxin, respectively. The rectangles mark the probable binding epitopes, which differ but are highly overlapping based on the internal binding. Although the sequences differ, the epitopes are related and therefore the complementary binding sites of the two toxins should be structurally and evolutionary related. The sequences are for GM1: Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ-Cer, and for GQ1b: NeuAcα8NeuAcα3 Galβ3GalNAcβ4(NeuAcα8NeuAcα3) Galβ4GlcβCer. For modelling conditions, see Table 4.

affinities, as for periplasmic transport proteins of bacteria, crystal structures of complexes of protein and monosaccharide have shown that all possible interaction points in the binding site have been used, including directed hydrogen bonding and van der Waals interaction (33).

It is possible to use multivalent inhibitors without resorption on mucosal surfaces. However, in most cases a drug has to be resorbed through membranes and therefore has to be small enough and resist rapid elimination.

First proof of the concept of anti-adhesion for therapy

Recently, results were published that proved for the first time in a clinically relevant situation that anti-adhesion is possible (24). New-born calves were infected with a lethal dose of diarrhoea-inducing *E. coli* K99, and when symptoms of diarrhoea appeared they were given an oral dose of 1 - 3 g of oligosaccharides prepared from cow blood plasma, which was sufficient for cure. In parallel we showed (30) that the active receptor sequence for this bacterium is most probably limited to NeuGcα3Gal, and saccharides with this sequence were probably only part of the given dose. In relation to the fact that the small intestine, which is the target for these bacteria, has a total mucosal surface in the range of a soccer ground, these results are very promising. Theoretically, if the dose given contained 10% (at the most) of the receptor-active sequence, and, in analogy with the Manα derivative discussed above, an efficient synthetic analogue was used, the active dose could be reduced to 100 - 300 micrograms per animal.

Drug design

Rational drug design is considered based on a three-dimensional knowledge of the receptor-binding protein site, most optimally the receptor-protein complex. Only one such case has been resolved so far for attachment complexes, namely the crystal structure of influenza virus haemagglutinin in complex with sialic acid (34). Interestingly, the receptor carbohydrate did produce less sharp diffraction, indicating a looser position, in line with the low-affinity interaction.

Although the information on crystal structures of such complexes cannot yet be obtained to allow computer-based modelling of optimized synthetic low-molecular receptor analogues, it is possible to approximate binding epitopes on receptor saccharides without structural knowledge of the protein. This we have named epitope dissection (13); it is based on the property of microbes to bind internally in the chain to produce families of isoreceptors (see discussion above). If the binding preferences to a sufficient number of isoreceptors are known, including positive and negative isoreceptors, computer-based molecular modelling of the isoreceptor sequences

may inform us of steric hindrances and other causes of changed interaction. This allows a conclusion to be drawn as to which side of the minimum common sequence the binding occurs and what effects neighbouring groups may have. A good example is the minimum epitope on isoreceptors of cholera toxin compared with isoreceptors of tetanus toxin (17, and to be published). In Fig. 8 proposed minimum epitopes have been indicated on molecular models of the optimal natural receptors for the two toxins, GM1 and GQ1b gangliosides, respectively. Of interest is the limited minimum requirement found on one side and internally in these molecules, and that the binding epitopes are highly overlapping, indicating a structural and evolutionary relation between the complementary binding sites of the two toxins. The toxins are thus members of the NeuAc-binding family as defined in Table 1. Therefore, probably only a slight change in the amino acid sequence of the receptor-binding site may explain the quite separate target cells (intestine and nerve) producing different symptoms (diarrhoea and tetanus). Theoretically, a synthetic analogue may be designed that will inhibit the binding of both toxins.

PERSPECTIVES

There is at present rapid development in the field of microbial interactions with animal cells based on protein-carbohydrate interactions. Important findings are the internal recognition of the receptor sequence and families of receptor-binding variants. In one single case, the concept of anti-adhesion therapy has been proven, and it is now technically possible to develop this field to encompass broad biomedical applications including therapy of human infections. Globally, there are major infectious diseases such as malaria and AIDS where there is growing evidence that protein-carbohydrate interactions are essential for the establishment of an infection.

ACKNOWLEDGEMENT

The work was supported by grants from the *Swedish Medical Research Council* and from *Symbicom Ltd.*

REFERENCES

1. *Bell, G. I., Dembo, M. & Bongrand, P.*: Cell adhesion. Competition between nonspecific repulsion and specific bonding. Biophys. J. *45*: 1051 - 1064, 1984.
2. *Bhat, S., Spitalnik, S. L., Gonzalez-Scarano, F. & Silberberg, D. H.*: Galactosyl ceramide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp 120. Proc. Natl. Acad. Sci. USA *88:* 7131 - 7134, 1991.
3. *Bock, K., Breimer, M. E., Brignole, A., Hansson, G. C., Karlsson, K.-A., Larson, G., Leffler, H., Samuelsson, B. E., Strömberg, N., Svanborg Edén, C. & Thurin, J.*: Specificity of binding of a strain of uropathogenic *Escherichia coli* to Galα1-4Gal containing *glycosphingolipids. J. Biol. Chem. 260:* 8545 - 8551, 1985.
4. *Firon, N., Ashkenazi, S., Mirelman, D., Ofek, I. & Sharon, N.*: Aromatic alpha glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated *Escherichia coli* to yeast and intestinal epithelial cells. Infect. Immun. *55:* 472 - 476, 1987.
5. *Gahmberg, C. & Hakomori, S.-I.*: Surface carbohydrates of hamster fibroblasts. II. Interaction of hamster NIL cells with *Ricinus communis* lectin and concanavalin A as revealed by a surface galactosyl label. J. Biol. Chem. *250:* 2447 - 2451, 1975.
6. *Gamian, A., Chomik, M., Laferrière, C. A. & Roy, R.*: Inhibition of influenza A virus hemagglutinin and induction of interferon by synthetic sialylated glycoconjugates. Can. J. Microbiol. *37:* 233 - 237, 1991.
7. *Glick, G. D. & Knowles, J. R.*: Molecular recognition of bivalent sialosides by influenza virus. J. Am. Chem. Soc. *113:* 4701 - 4703, 1991.
8. *Hansson, G. C., Karlsson, K.-A., Larson, G., Strömberg, N., Thurin, J., Örvell, C. Norrby, E.*: A novel approach to the study of glycolipid receptors for viruses: binding of Sendai virus to thin-layer chromatograms. FEBS Lett. *170:* 15- 18, 1984.
9. *Hansson, G. C., Karlsson, K.-A., Larson, G., Strömberg, N. & Thurin, J.*: Carbohydrate-specific adhesion of bacteria to thin-layer chromatograms: a rationalized approach to the study of host cell glycolipid receptors. Anal. Biochem. *146:* 158 - 163, 1985.
10. *Harouse, J. M., Bhat, S., Spitalnik, S. L., Laughlin, M., Stefano, K., Silberberg, D. H. & Gonzalez-Scarano, F.*: Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. Science *253:* 320 - 323, 1991.
11. *Jimenez-Lucho, V., Ginsburg, V. & Krivan, H. C.: Cryptococcus neoformans, Candida albicans*, and other fungi bind specifically to the glycosphingolipid lactosylceramide (Galβ1-4Glcβ1-1Cer), a possible adhesion receptor for yeasts. Infect. Immun. *58:* 2085 - 2090, 1990.
12. *Karlsson, K.-A.*: Glycosphingolipids and surface membranes. In: *Chapman, D.* (Ed.): Biological Membranes 4, pp. 1 - 74, (Academic Press, London), 1982.
13. *Karlsson, K.-A.*: Animal glycosphingolipids as membrane attachment sites for bacteria. Annu. Rev. Biochem. *58:* 309 - 350, 1989.

14. *Karlsson, K.-A.:* Glycobiology: a growing field for drug design. Trends Pharmacol. Sci. *12:* 265 - 272, 1991.
15. *Karlsson, K.-A. & Strömberg, N.:* Overlay and solid-phase analysis of glycolipid receptors for bacteria and viruses. Meth. Enzymol. *138:* 220 - 232, 1987.
16. *Karlsson, K.-A., Norrby, E. & Wadell, G.:* Antiviral agents. In: United States Patent, no. 4,980,462, 1990.
17. *Karlsson, K.-A., Ångström, J. & Teneberg, S.:* On the characteristics of carbohydrate receptors for bacterial toxins: aspects of the analysis of binding epitopes. In: *Alouf, J. E. & Freer, J. H.* (Eds.): Sourcebook of Bacterial Protein Toxins, pp. 435-443, (Academic Press, London), 1991.
18. *Karlsson, K.-A., Abul Milh, M., Ångström, J., Bergström, J., Dezfoolian, H., Lanne, B., Leonardsson, I. & Teneberg, S.:* Membrane proximity and internal binding in the microbial recognition of host cell glycolipids. A conceptual discussion. In: *Korhonen, T., Hovi, T. & Mäkelä, P. H.* (Eds.): Molecular recognition in host parasite interactions, in press, (Plenum Press), 1992.
19. *Lindberg, A. A., Brown, J. E., Strömberg, N., Westling-Ryd, M., Schultz, J. E. & Karlsson, K.-A.:* Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J. Biol. Chem. *262:* 1779 - 1785, 1987.
20. *Lindstedt, R., Larson, G., Falk, P., Jodal, U., Leffler, H. & Svanborg, C.:* The receptor repertoire defines the host range for attaching *Escherichia coli* strains that recognize globo-A. Infect. Immun. *59:* 1086 - 1092, 1991.
21. *Marklund, B.-I., Tennent, J. M., Garcia, E., Hamers, A., Båga, M., Lindberg, F., Gaastra, W. & Normark, S.:* Horizontal gene transfer of the *E. coli pap* and *prs* operons as a mechanism for the development of tissue specific adhesive properties. Manuscript, 1991.
22. *Matrosovich, M. N., Mochalova, L. V., Marinina, V. P., Byramova, N. E. & Bovin, N. V.:* Synthetic polymeric sialoside inhibitors of influenza virus receptor-binding activity. FEBS Lett. *272:* 209 - 212, 1990.
23. *Mayo, S. L., Olafson, B. D. & Goddard III, W. A.:* DREIDING: A generic force field for molecular simulations. J. Phys. Chem. *94:* 8897 - 8909, 1990.
24. *Mouricout, M., Petit, J. M., Carias, J. R. & Julien, R.:* Glycoprotein glycans that inhibit adhesion of *Escherichia coli* mediated by K99 fimbriae: Treatment of experimental colibacillosis. Infect. Immun. *58:* 98 - 106, 1990.
25. *Sabesan, S., Duus, J. Ö., Domaille, P., Kelm, S. & Paulson, J. C.:* Synthesis of cluster sialoside inhibitors for influenza virus. J. Am. Chem. Soc. *113:* 5865 - 5866, 1991.
26. *Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R. & Tuomanen, E.:* Pertussis toxin has eukaryotic-like carbohydrate recognition domains. Proc. Natl. Acad. Sci. USA *89:* 118-122, 1992.
27. *Strömberg, N. & Karlsson, K.-A.:* Characterization of the binding of *Propionibacterium granulosum* to glycosphingolipids adsorbed on surfaces. An apparent recognition of lactose which is dependent on ceramide structure. J. Biol. Chem. *265:* 11244 - 11250, 1990.
28. Strömberg, N. & Karlsson, K.-A.: Characterization of the binding of *Actinomyces naeslundii* and *Actinomyces viscosus* to glycosphingolipids, using a solid-phase overlay approach: J. Biol. Chem. *265:* 11251 - 11258, 1990.
29. *Strömberg, N., Marklund, B.-I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K.-A. & Normark, S.:* Host-specificity of uropathogenic *E. coli* depends on differences in binding specificity to Galα1-4Gal-containing isoreceptors. EMBO J. *9:* 2001 - 2010, 1990.
30. *Teneberg, S., Willemsen, P., de Graaf, F. K. & Karlsson, K.-A.:* Receptor-active glycolipids of epithelial cells of the small intestine of young and adult pigs in relation to susceptibility to infection with *Escherichia coli* K99. FEBS Lett. *263:* 10 - 14, 1990.
31. *Thurin, J.:* Binding sites of monoclonal anti-carbohydrate antibodies. Current Topics Microbiol. Immun. *139:* 59 - 79, 1988.
32. *Toogood, P. L., Galliker, P. K., Glick, G. D. & Knowles, J. K.:* Monovalent sialosides that bind tightly to influenza A virus. J. Med. Chem. *34:* 3138 - 3140, 1991.
33. *Vyas, N. K.:* Atomic features of protein-carbohydrate interactions. Curr. Opin. Struct. Biol. *1:* 732 - 740, 1991.
34. *Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J. & Wiley, D. C.:* Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature *333:* 426 - 431, 1988.